The Effects of Antibacterial Substances from Foods and Lactic Acid Bacteria on Lactococcus garvieae

著者	菊池 葵, 田名部 彩花, 古倉 あゆみ, 三浦 涼平, 西村 順子
著者別名	KIKUCHI Aoi, TANABU Ayaka, FURUKURA Ayumi, MIURA Ryohei, NISHIMURA Junko
journal or publication title	The Bulletin of Institute for Promotion of Community Industrial Activities Hachinohe Institute of Technology
volume	16
page range	7-12
year	2018-03-30
URL	http://id.nii.ac.jp/1078/00003848/

Lactococcus garvieae に対する食品および乳酸菌由来抗菌性物質の影響

菊池葵 *・田名部彩花 *・古倉あゆみ *・三浦涼平 *・西村順子 *

論文要約

乳酸菌の一種の *Lactococcus garvieae* は，魚病や乳房炎の起因菌としてのみならず，ヒトに対しても不顕性感染を引き起こしていることが判明している。本菌に対して，食品由来抗菌性物質である茶カテキン 4 種（エピカテキン，エピカテキンガレート，エピガロカテキン，エピガロカテキンガレート）と，乳酸菌由来バクテリオシンのナイシンおよびガセリシンの抗菌効果について，寒天拡散法とマイクロプレート法を用いて検証した。その結果，緑茶カテキンでは抗菌効果が認められなかったが，乳酸菌由来バクテリオシンでは条件次第で抗菌効果が認められた。今回使用した乳酸菌由来バクテリオシンのナイシンは，正に帯電して細胞膜に静電結合して孔を形成し，ATP などの細胞内容物を細胞外に流出させて抗菌活性を示すことから，*Lc. garvieae* の細胞膜は帯電性の抗菌性物質の影響を受けやすい構造を有していることが示唆された。また 2 種の方法での活性の違いがみられ，抗菌活性は自由水の割合や酸素分圧に影響されるのではないかと考えられた。今後，より詳細な研究を行い，安全な抗菌性物質の開発を考えている。

キーワード：ガルビエ菌，緑茶カテキン，ナイシン，ガセリシン，抗菌活性

The Effects of Antibacterial Substances from Foods and Lactic Acid Bacteria on *Lactococcus garvieae*

Aoi Kikuchi*, Ayaka Tanabu*, Ayumi Furukura*, Ryohei Miura* and Junko Nishimura*

ABSTRACT

It has been demonstrated that *Lactococcus garvieae* causes not only diseases in fish but also mastitis in cows and subclinical infections in humans. In this study, we examined the antibacterial effects of green tea catechins and bacteriocins produced from lactic acid bacteria on *Lc. garvieae*. The results showed that an antibacterial effect was recognized on bacteriocin from lactic acid bacteria, *i.e.*, nisin in the agar well diffusion assay and *Lactobacillus gasseri* LA39 supernatant in the microplate assay, whereas green tea catechins didn't. It was suggested that the cell membrane of *Lc. garvieae* was easily affected by the electrostatic charged antibacterial substances. Furthermore, the antibacterial effects differed between agar well diffusion and microplate assay, so the ration of free water and oxygen tension might be affected.

Keywords: *Lactococcus garvieae, green tea catechin, nisin, gassericin, antibacterial activity*

平成 29 年 12 月 28 日

* 八戸工業大学工学部バイオ環境工学科

1. Introduction

It is well known that lactic acid bacteria is the general term for bacteria, which can produce lactic acid as the final product of carbohydrate metabolism, and contributes to health as probiotics, on the other hand, there are some strains indicating the pathogenicity.

Lactococcus garvieae, which is a type of lactic acid bacteria, not only causes epidemic infectious disease in cultured fish, but also infects latency in humans [1]. It is considered that capsular polysaccharide or exopolysaccharide encoding on plasmid is related to the pathogenicity [1]. The clinical isolated strain possesses five plasmids, and three of them relate to bacteriocin production [2]. At present, there is concern about outbreaks of hospital infection by healthy carrier humans and the presence of multiple varieties of drug resistant bacteria [1].

Recently, there has been interest in the function of foods. Catechins, which is including in Japanese green tea, has been authenticated as Generally Recognized As Safe (GRAS). Representative Japanese green tea catechins are epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate, and it been proven that these catechins have antibacterial and antivirus properties and have the effect of inhibiting toxin production [3]. The antibacterial activities of catechins are mainly reported against gram-positive bacteria such as *Staphylococcus aureus, Bacillus cereus, Streptococcus mutans,* and *Str. sanguinis*[4, 5]. It has been suggested that the sensitivity for catechins depends on the chemical structure of the bacterial cell surface [6, 7].

The anti-bacterial function of lactic acid bacteria has also been a focus for the safety of food. Peptides or proteins, which indicate the antibacterial effects on related strain and synthesized on ribosome, are called "bacteriocin". Especially, nisin produced from *Lactococcus lactis* subsp. *lactis* has been used as a food additive in over 50 countries in the world and was officially authorized in 2009 in Japan [8]. It has been found that nisin could effectively inhibit the growth of gram-positive food pathogenic bacteria such as *Bacillus, Clostridium, Staphylococcus,* and *Listeria* genus[9-11], resist high temperatures and low pH, and is easily digested in stomach and intestinal tract[8, 12]. Under these specific conditions, it is considered that resistant bacteria rarely appear.

Nevertheless, the bacteriocin, with the exception of nisin, are rarely used in the industry at present. If they become available, the control of harmful bacteria will be easier. In this study, we examined the effect of green tea catechins and bacteriosins from lactic acid bacteria to prevent the infection of *Lc. garveae* and aimed for the establishment of an effective antibacterial approach.

2. Materials and Methods

2.1 Bacterial strain and culture

Lactococcus garvieae JCM10343^T was purchased from Japan Collection of Microorganisms (Tsukuba, Japan). This strain was cultivated with MRS broth (Difco™ Lactobacilli MRS broth, BD, NJ, USA) and stored at -80℃ until use.

The strain culture was inoculated into 5 ml of MRS broth with a ratio of 1% (v/v) and cultivated at 30℃ for 24 hr. This procedure was repeated twice before the examination.

2.2 Preparation of sample solutions

Epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC), and epigallocatechin gallate (EGCg) were purchased from Wako Industrial Co. Ltd. (Osaka, Japan). Catechins were dissolved into distilled water with a concentration of 1 mg/ml, and sterilized through a 0.20μm pore size membrane filter (DISMIC 13CP, ADVANTEC, Tokyo, Japan).

Nisin (from *Lactococcus lactis,* Sigma-Aldrich, USA) was dissolved into 20% ethanol with a concentration of 2 mg/10ml, and stored at 4℃ after sterilizing filtration.

Lactobacillus gasseri LA39 and LA158 were cultured twice in MRS broth and inoculated 1% (v/v) was inoculated into 5 ml of MRS broth. After cultivation (37℃, 24 hr), the culture was centrifuged (5,000 rpm, 10 min, room temperature) to remove bacterial cells. The culture supernatants were sterilized by filtration and stored at 4℃.

Just before the experiment, each solution was diluted serially using distilled water for catechins, 20% ethanol for nisin, and 0.85% NaCl for *Lb. gasseri* supernatant.

2.3 Agar well diffusion assay

Anti-bacterial activity was determined with the agar well diffusion method. According to previous reports[13], MRS agar plates were overlaid with MRS soft agar (0.75%) lawn prepared *Lc. garvieae* JCM10343^T and wells (d.m. 5.5 mm) were made, then 65 μl of the sample solutions was put into each well.

The plates were incubated at 30℃ for 24 hr and a

hallo formation inhibiting the growth of *Lc. garvieae* JCM10343^T was observed with direct transmitting lighting installation.

2.4 Microplate assay

Anti-bacterial activity was also determined using the microplate method. MRS broth (160 μl) was put into a well on a 96-well microplate (Sumitomo Bakelite Co. Ltd., Tokyo, Japan), the culture of *Lc. garvieae* JCM10343^T (20 μl) and each sample solutions (20 μl) were added to the well, and the microplate was incubated at 30℃ for 24 hr.

After cultivation, the turbidity, which had been diluted 10 times with distilled water, was measured at 600 nm with a spectrophotometer (U-1800 Hitachi, Tokyo, Japan).

2.5 Statistics

The turbidity with a triplicate of each sample was measured. And then, the means, standard deviations, and significant difference by ANOVA analysis and multiple comparison authorization (Sheffe's) were calculated (* <0.05).

3. Results and Discussion

3.1 The effect of green tea catechins on *Lc. garvieae* JCM10343^T

The anti-microbial activity of green tea chatechins was evaluated by agar well diffusion and microplate assay (Fig. 1-3). On both assays, inhibitions with significant differences were not observed at all. These results showed that the anti-bacterial activity of green tea catechins could not be expected for *Lc. garvieae*. It has been demonstrated that green tea catechins acts to Lys-type peptidoglycan such as *Lactobacillus acidophilus,* while it had no almost antibacterial effect to DAP-type peptidoglycan on *Lactobacillus plantarum*[14]. It was considered that green tea catechins were not able to exhibit antibacterial activity because crosslink peptide of peptidoglycan on *Lc. garvieae* combined with Lys-Ala-Gly-Ala, not generally L-Lys-D-Asp[12, 15]. In addition, cloudy circles were recognized at the concentration of 1-2 AU/ml ECg on the agar well diffusion assay. This phenomenon was observed before[16], and it seemed that MRS medium ingredient and ECg caused aggregation.

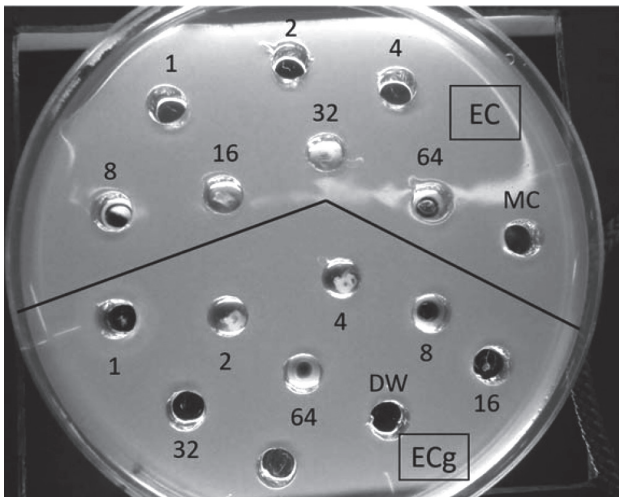


Figure 1 Anti-microbial activity of epicatechin and epicatechin gallate by agar well diffusion assay (Epicatechin (EC), Epicatechin gallate (ECg))

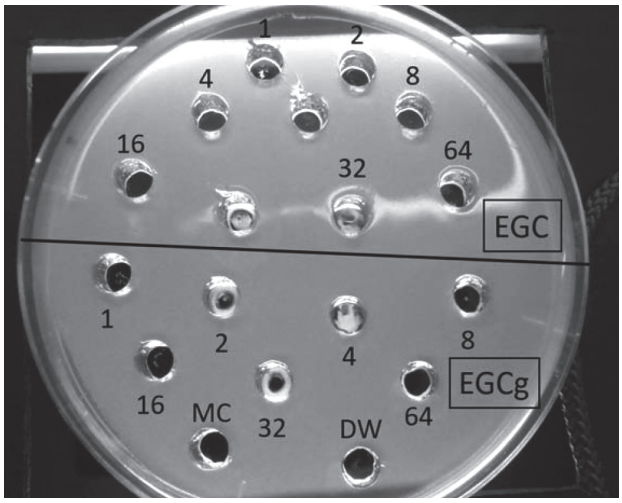
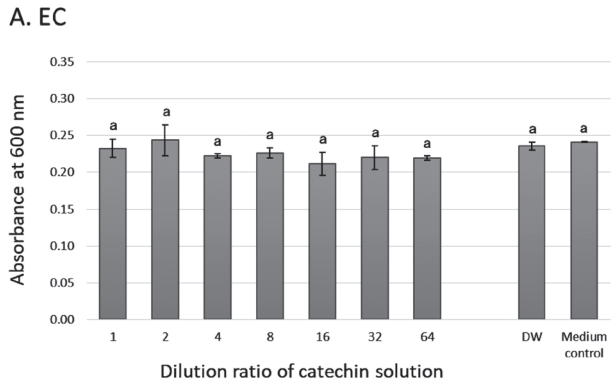


Figure 2 Anti-microbial activity of epigallocatechin and epigallocatechin gallate by agar well diffusion assay (Epigallocatechin (EGC), Epigallocatechin gallate (EGCg))



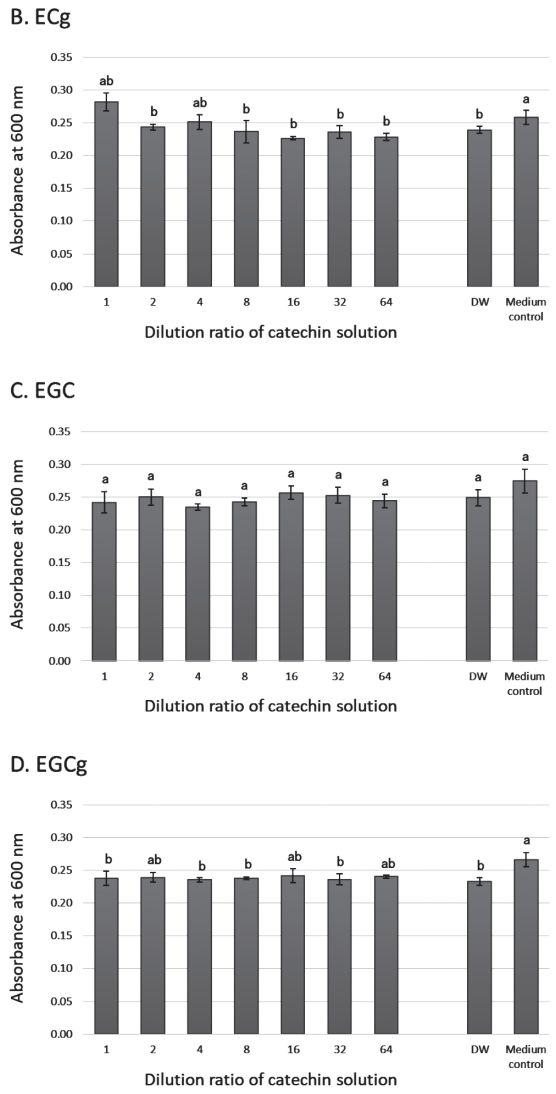


Figure 3　Anti-microbial activity of green tea catechins by microplate assay
（A : Epicatechin（EC）, B : Epicatechin gallate（ECg）, C : Epigallocatechin（EGC）, D : Epigallocatechin gallate（EGCg））

3.2 The effects of bacteriocins produced lactic acid bacteria on *Lc. garvieae* JCM10343^{T}

The anti-microbial activity of bacteriocins produced from lactic acid bacteria was evaluated by agar well diffusion and microplate assay（Fig. 4-7）. In the agar well diffusion assay, anti-bacterial activity was found in nisin（2 AU/ml）, nevertheless neither *Lb. gasseri* LA39 nor LA158. On the other hand, anti-microbial activity of nisin didn't determine significant differences in the microplate assay. In addition, *Lb. gasseri* LA39 supernatant indicated anti-bacterial activity on microplate assay（2 AU/ml）.

It has been demonstrated that there are loops in nisin molecules, because unusual amino acids such as rantionin exists within its structure. Gassericin A produced from *Lb. gasseri* LA39 is a cyclic peptide with strong hydrophobicity[13]. Furthermore, nisin takes a positive charge in the solution, perforates bacterial cell membrane, and leads to run off cellular contents such as ATP [2]. Two types of bacteriocin, which had anti-bacterial activity, might act to cell membrane of *Lc. garvieae* by the biochemical characteristic similarity. In addition, it was suggested that anti-bacterial differences between the agar well diffusion assay and the microplate assay were related to the proportions of free water and oxygen partial pressure.

Based on these results, it is considered that *Lc. garvieae* is able to resist existing anti-bacterial substances on behalf of the chemical characterization of its peptide-glycan or cell membrane. To develop secure antibiotics, it is necessary to conduct research in detail on bacterial mechanisms and bacterial cell surface chemical structures.

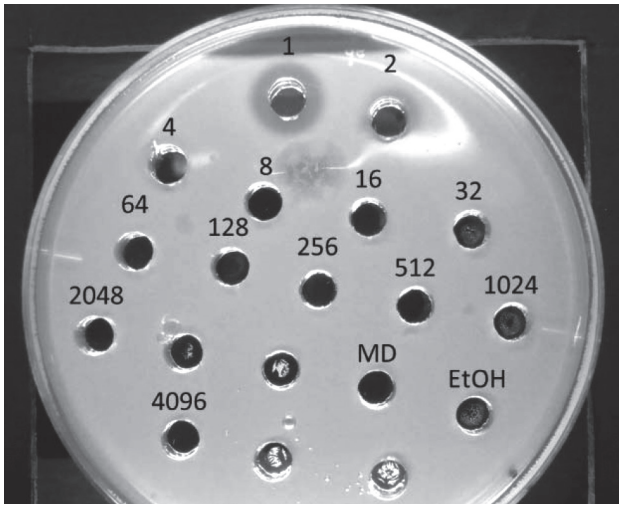


Figure 4　Anti-microbial activity of nisin in agar well diffusion assay

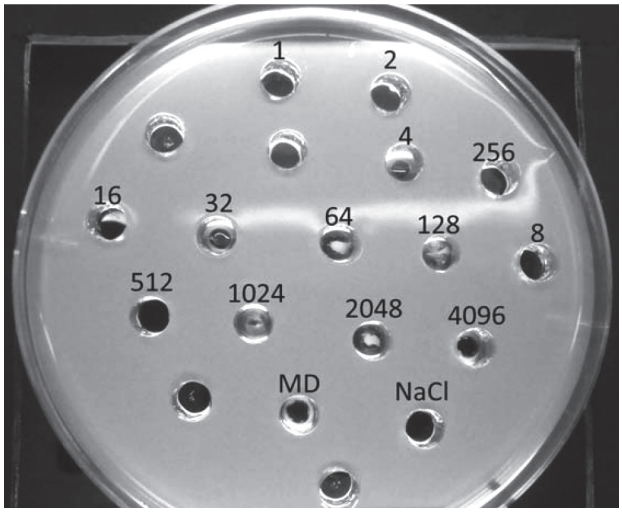


Figure 5　Anti-microbial activity of *Lb. gasseri* LA39 supernatant in agar well diffusion assay

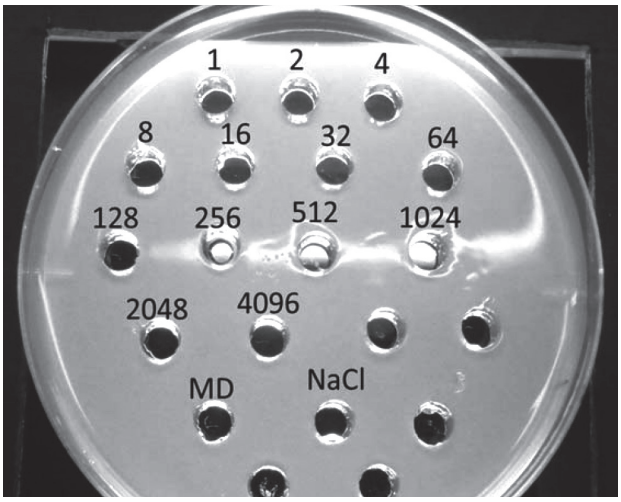


Figure 6 Anti-microbial activity of *Lb. gasseri* LA158 supernatant in agar well diffusion assay

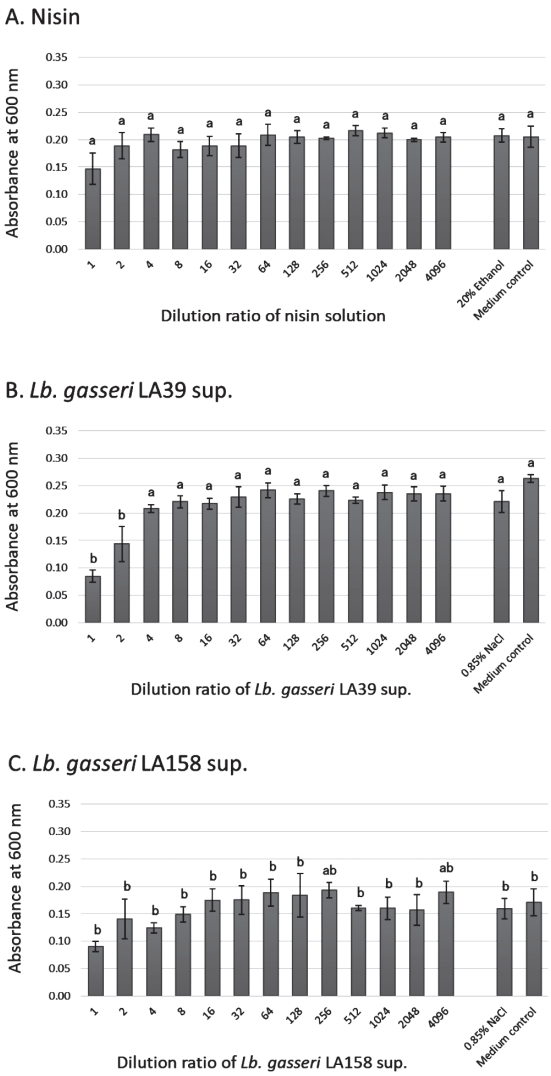


Figure 7 Anti-microbial activity of bacteriocins in microplate assay
（A : nisin, B : *Lb. gasseri* LA39 supernatant, C : *Lb. gasseri* LA158 supernatant）

4. Conclusion

The antimicrobial effects of green tea catechin and bacteriocin produced from lactic acid bacteria on *Lactococcus garvieae* were investigated. As a result, bacteriocin from lactic acid bacteria showed antimicrobial activity depending on a condition, whereas green tea cathechins didn't. On this study, it was suggested that the antimicrobial activity to *Lc. garvieae* was influenced chemical characteristics of bacterial surface and ratio of free water and/or oxygen in environmental condition.

5. Acknowledgement

We are grateful to Dr. Yoshiyuki Tanaka for statistical analysis in this study.

References

1）岡本陽 , 荒川宣親：ヒトにおける感染症起因菌としての *Lactococcus garvieae,* 日本臨床微生物学雑誌 , 22（1）, 1-12, 2012.
2）M. Aguado-Urda, *et al*.: Characterization of plasmids in a human clinical strain of *Lactococcus garvieae,* PLoS ONE, 7（6）, e40119, 2012.
3）www.catechin-society.com/iroha.html
4）原 征彦：茶カテキン類の機能性とそれらの応用例 , 日本食品保蔵科学会誌 , 26（1）, 47-54, 2000.
5）日下部 修介ら：カテキン粉末緑茶の口腔内細菌に及ぼす影響 , 日本歯科保存学雑誌, 56（4）, 277-284, 2013.
6）Functional anatomy of prokalyotic and eukaryotic cells, www2. sunysuffolk. edu/czuraa/BIO244LectureMaterials/BIO244Chapter4Notes.pdf
7）www.glycoforum.gr.jp/science/word/immunity/IS-A01J.html
8）益田時光 , 善藤威史 , 園元謙二：ナイシン - 類稀な抗菌物質 , ミルクサイエンス , 59（1）, 59-65, 2010.
9）石橋直樹, 善藤威史, 園元謙二：乳酸菌バクテリオシン - 戦略的な探索・発見・活用とゼロエミッション PJ まで -, 日本乳酸菌学会誌, 22（1）, 38-48, 2011.
10）www.aichi-inst.jp/shokuhin/research/report/food_2004_01.pdf
11）www.food-tokachi.jp/research/h18result/cheese-nisin_h16-18.pdf
12）乳酸菌とビフィズス菌のサイエンス , 日本乳酸菌学会編 , 京都大学出版会 , 309-324, 2010.
13）Y. Kawai, *et al*: Gassericin A; an uncommon cyclic bacteriocin produced by *Lactobacillus gasseri* LA39

linked at N- and C-terminal ends, Bioscience Biotechnology and Biochemistry, 62（12）, 2438-2440, 1998.
14）西山隆造，尾崎道雄：フェノール関連物質の微生物生育阻害作用について，49-71，1991. www.teikyo-jc.ac.jp/jyoho/periodical_pdf
15）K.H.Schleifer, *et al* : Transfer of *Streptococcus lactis* and related Streptococci to the genus *Lactococcus* gen. nov., Systematic and Applied Microbiology, 6, 183-195, 1985.
16）阿部光一，前澤冬樹：ヒト乳児糞便由来 *Lactobacillus gasseri* のバクテリオシン生産能評価と茶カテキンの影響について，平成 26 年度八戸工業大学工学部バイオ環境工学科卒業研究論文 .